

FORM PTO-1300 (REV. 11-2000)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER 30448.77USW1	
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371				U.S. APPLICATION NO. (if known, see 37 CFR 1.5) 09/936665	
INTERNATIONAL APPLICATION NO. PCT/US00/06232		INTERNATIONAL FILING DATE March 3, 2000		PRIORITY DATE CLAIMED March 3, 1999	
TITLE OF INVENTION ADIPOSE-DERIVED STEM CELLS AND LATTICES					
APPLICANT(S) FOR DO/EO/US Marc H. Hedrick et al.					
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:					
1. <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. 371. 2. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371. 3. <input checked="" type="checkbox"/> This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below. 4. <input checked="" type="checkbox"/> The US has been elected by the expiration of 19 months from the priority date (Article 31). 5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2)) a. <input checked="" type="checkbox"/> is attached hereto (required only if not communicated by the International Bureau). b. <input type="checkbox"/> has been communicated by the International Bureau. c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US). 6. <input checked="" type="checkbox"/> An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)). a. <input type="checkbox"/> is attached hereto. b. <input checked="" type="checkbox"/> has been previously submitted under 35 U.S.C. 154(d)(4). 7. <input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)) a. <input checked="" type="checkbox"/> are attached hereto (required only if not communicated by the International Bureau). b. <input type="checkbox"/> have been communicated by the International Bureau. c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired. d. <input type="checkbox"/> have not been made and will not be made. 8. <input type="checkbox"/> An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)). 9. <input checked="" type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). 10. <input type="checkbox"/> An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)). Items 11 to 20 below concern document(s) or information included: 11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98. 12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included. 13. <input checked="" type="checkbox"/> A FIRST preliminary amendment. 14. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment. 15. <input type="checkbox"/> A substitute specification. 16. <input type="checkbox"/> A change of power of attorney and/or address letter. 17. <input type="checkbox"/> A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825. 18. <input type="checkbox"/> A second copy of the published international application under 35 U.S.C. 154(d)(4). 19. <input type="checkbox"/> A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4). 20. <input checked="" type="checkbox"/> Other items or information: Transmittal sheets in duplicate, Postcard, Application Data Sheet, International Search Report (English), Check, Published International Application No. WO00/53795; PCT Written Opinion dated April 19, 2001 and Response to PCT Written Opinion dated May 18, 2001.					

21. ☒ The following fees are submitted:**BASIC NATIONAL FEE (37 CFR 1.492(a) (1) - (5)):**

Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO. \$1000.00

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO \$860.00

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$710.00

International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) \$690.00

International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) \$100.00

ENTER APPROPRIATE BASIC FEE AMOUNT =

CALCULATIONS PTO USE ONLY

\$ 710.00

Surcharge of \$130.00 for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).

\$

CLAIMS

NUMBER FILED

NUMBER EXTRA

RATE

\$

Total claims 20 - 20 = 0 x \$18.00 \$ 0

Independent claims 1 - 3 = 0 x \$80.00 \$ 0

MULTIPLE DEPENDENT CLAIM(S) (if applicable) + \$270.00 \$ 270.00

TOTAL OF ABOVE CALCULATIONS = \$ 980.00

☒ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2. \$ 490.00

SUBTOTAL = \$ 490.00

Processing fee of \$130.00 for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)).

\$

TOTAL NATIONAL FEE = \$ 490.00

Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property + \$ 0.00

TOTAL FEES ENCLOSED = \$ 490.00

Amount to be refunded: \$

charged: \$

- a. ☒ A check in the amount of \$ 490.00 to cover the above fees is enclosed.
- b. ☐ Please charge my Deposit Account No. 50-0306 in the amount of \$ to cover the above fees. A duplicate copy of this sheet is enclosed.
- c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 50-0306. A duplicate copy of this sheet is enclosed.
- d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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REGISTRATION NUMBER

531 Rec'd PCT

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10 SEP 2001

Dkt. 30448.77USW1 SLM/AHB/RDG

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: Marc H. Hedrick et al.

Serial No.: not yet known

Examiner: not yet known

Filed: herewith

Group Art Unit: not yet known

Title: ADIPOSE-DERIVED STEM CELLS AND LATTICES

35 North Arroyo Parkway
Pasadena, California 91103
September 10, 2001

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

PRELIMINARY AMENDMENT

Please amend the subject application as follows.

IN THE CLAIMS:

Please cancel claims 8-138 without prejudice to pursue the subject matter of these claims in a continuation application to be filed in the future.

Applicants: Marc H. Hedrick et al.,
U.S. Serial No. not yet known
Filed: herewith
Page: 2

No fee is deemed necessary in connection with the filing of this Preliminary Amendment. If any fee is necessary, the Patent Office is authorized to charge any additional fee to Deposit Account No. 50-0306.

Respectfully submitted,



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ADIPOSE-DERIVED STEM CELLS AND LATTICES

BACKGROUND OF THE INVENTION

In recent years, the identification of mesenchymal stem cells, chiefly
5 obtained from bone marrow, has led to advances in tissue regrowth and
differentiation. Such cells are pluripotent cells found in bone marrow and
periosteum, and they are capable of differentiating into various mesenchymal or
connective tissues. For example, such bone-marrow derived stem cells can be
induced to develop into myocytes upon exposure to agents such as 5-azacytidine
10 (Wakitani et al., *Muscle Nerve*, 18(12), 1417-26 (1995)). It has been suggested
that such cells are useful for repair of tissues such as cartilage, fat, and bone (see,
e.g., U.S. Patents 5,908,784, 5,906,934, 5,827,740, 5,827,735), and that they also
have applications through genetic modification (see, e.g., 5,591,625). While the
identification of such cells has led to advances in tissue regrowth and
15 differentiation, the use of such cells is hampered by several technical hurdles.
One drawback to the use of such cells is that they are very rare (representing as
few as 1/2,000,000 cells), making any process for obtaining and isolating them
difficult and costly. Of course, bone marrow harvest is universally painful to the
donor. Moreover, such cells are difficult to culture without inducing
20 differentiation, unless specifically screened sera lots are used, adding further cost
and labor to the use of such stem cells. Thus, there is a need for a more readily
available source for pluripotent stem cells, particularly cells that can be cultured
without the requirement for costly prescreening of culture materials.

Other advances in tissue engineering have shown that cells can be grown in
25 specially-defined cultures to produce three-dimensional structures. Spacial definition
typically is achieved by using various acellular lattices or matrices to support and
guide cell growth and differentiation. While this technique is still in its infancy,
experiments in animal models have demonstrated that it is possible to employ various
acellular lattice materials to regenerate whole tissues (see, e.g., Probst et al. *BJU Int.*,
30 85(3), 362-7 (2000)). A suitable lattice material is secreted extracellular matrix
material isolated from tumor cell lines (e.g., Engelbreth-Holm-Swarm tumor secreted
matrix - "matrigel"). This material contains type IV collagen and growth factors,
and provides an excellent substrate for cell growth (see, e.g., Vukicevic et al., *Exp.*
Cell Res, 202(1), 1-8 (1992)). However, as this material also facilitates the malignant
35 transformation of some cells (see, e.g., Fridman, et al., *Int. J. Cancer*, 51(5), 740-44
(1992)), it is not suitable for clinical application. While other artificial lattices have
been developed, these can prove toxic either to cells or to patients when used *in vivo*.

Accordingly, there remains a need for a lattice material suitable for use as a substrate in culturing and growing populations of cells.

BRIEF SUMMARY OF THE INVENTION

5 The present invention provides adipose-derived stem cells and lattices. In one aspect, the present invention provides a lipo-derived stem cell substantially free of adipocytes and red blood cells and clonal populations of connective tissue stem cells. The cells can be employed, alone or within biologically-compatible compositions, to generate differentiated tissues and structures, both *in vivo* and *in vitro*. Additionally, the cells can be expanded and cultured to produce hormones and to provide conditioned culture media for supporting the growth and expansion of other cell populations. In another aspect, the present invention provides a lipo-derived lattice substantially devoid of cells, which includes extracellular matrix material from adipose tissue. The lattice can be used as a substrate to facilitate the growth and differentiation of cells, whether *in vivo* or *in vitro*, into anlagen or even mature tissues or structures.

Considering how plentiful adipose tissue is, the inventive cells and lattice represent a ready source of pluripotent stem cells. Moreover, because the cells can be passaged in culture in an undifferentiated state under culture conditions not requiring prescreened lots of serum, the inventive cells can be maintained with considerably less expense than other types of stem cells. These and other advantages of the present invention, as well as additional inventive features, will be apparent from the accompanying drawings and in the following detailed description.

DETAILED DESCRIPTION OF THE INVENTION

25 One aspect of the invention pertains to a lipo-derived stem cell. Preferably, the stem cell is substantially free of other cell types (e.g., adipocytes, red blood cells, other stromal cells, etc.) and extracellular matrix material; more preferably, the stem cell is completely free of such other cell types and matrix material. Preferably, the inventive cell is derived from the adipose tissue of a primate, and more preferably a higher primate (e.g., a baboon or ape). Typically, the inventive cell will be derived from human adipose tissue, using methods such as described herein.

While the inventive cell can be any type of stem cell, for use in tissue engineering, desirably the cell is of mesodermal origin. Typically such cells, when isolated, retain two or more mesodermal or mesenchymal developmental phenotypes (i.e., they are pluripotent). In particular, such cells generally have the capacity to develop into mesodermal tissues, such as mature adipose tissue, bone, various tissues

of the heart (e.g., pericardium, epicardium, epimyocardium, myocardium, pericardium, valve tissue, etc.), dermal connective tissue, hemangial tissues (e.g., corpuscles, endocardium, vascular epithelium, etc.), muscle tissues (including skeletal muscles, cardiac muscles, smooth muscles, etc.), urogenital tissues (e.g., kidney, pronephros, meta- and meso-nephric ducts, metanephric diverticulum, ureters, renal pelvis, collecting tubules, epithelium of the female reproductive structures (particularly the oviducts, uterus, and vagina)), pleural and peritoneal tissues, viscera, mesodermal glandular tissues (e.g., adrenal cortex tissues), and stromal tissues (e.g., bone marrow). Of course, inasmuch as the cell can retain potential to develop into mature cells, it also can realize its developmental phenotypic potential by differentiating into an appropriate precursor cell (e.g., a preadipocyte, a premyocyte, a preosteocyte, etc.). Also, depending on the culture conditions, the cells can also exhibit developmental phenotypes such as embryonic, fetal, hematopoietic, neurogenic, or neuralgiagenic developmental phenotypes. In this sense, the inventive cell can have two or more developmental phenotypes such as adipogenic, chondrogenic, cardiogenic, dermatogenic, hematopoietic, hemangiogenic, myogenic, nephrogenic, neurogenic, neuralgiagenic, urogenitogenic, osteogenic, pericardiogenic, peritoneogenic, pleurogenic, splanchnogenic, and stromal developmental phenotypes. While such cells can retain two or more of these developmental phenotypes, preferably, such cells have three or more such developmental phenotypes (e.g., four or more mesodermal or mesenchymal developmental phenotypes), and some types of inventive stem cells have a potential to acquire any mesodermal phenotype through the process of differentiation.

The inventive stem cell can be obtained from adipose tissue by any suitable method. A first step in any such method requires the isolation of adipose tissue from the source animal. The animal can be alive or dead, so long as adipose stromal cells within the animal are viable. Typically, human adipose stromal cells are obtained from living donors, using well-recognized protocols such as surgical or suction lipectomy. Indeed, as liposuction procedures are so common, liposuction effluent is a particularly preferred source from which the inventive cells can be derived.

However derived, the adipose tissue is processed to separate stem cells from the remainder of the material. In one protocol, the adipose tissue is washed with physiologically-compatible saline solution (e.g., phosphate buffered saline (PBS)) and then vigorously agitated and left to settle, a step that removes loose matter (e.g., damaged tissue, blood, erythrocytes, etc.) from the adipose tissue. Thus, the washing and settling steps generally are repeated until the supernatant is relatively clear of debris.

The remaining cells generally will be present in lumps of various size, and the protocol proceeds using steps gauged to degrade the gross structure while minimizing damage to the cells themselves. One method of achieving this end is to treat the washed lumps of cells with an enzyme that weakens or destroys bonds
 5 between cells (e.g., collagenase, dispase, trypsin, etc.). The amount and duration of such enzymatic treatment will vary, depending on the conditions employed, but the use of such enzymes is generally known in the art. Alternatively or in conjunction with such enzymatic treatment, the lumps of cells can be degraded using other treatments, such as mechanical agitation, sonic energy, thermal energy, etc. If
 10 degradation is accomplished by enzymatic methods, it is desirable to neutralize the enzyme following a suitable period, to minimize deleterious effects on the cells.

The degradation step typically produces a slurry or suspension of aggregated cells (generally liposomes) and a fluid fraction containing generally free stromal cells (e.g., red blood cells, smooth muscle cells, endothelial cells, fibroblast cells, and stem
 15 cells). The next stage in the separation process is to separate the aggregated cells from the stromal cells. This can be accomplished by centrifugation, which forces the stromal cells into a pellet covered by a supernatant. The supernatant then can be discarded and the pellet suspended in a physiologically-compatible fluid. Moreover, the suspended cells typically include erythrocytes, and in most protocols it is
 20 desirable to lyse these. Methods for selectively lysing erythrocytes are known in the art, and any suitable protocol can be employed (e.g., incubation in a hyper- or hypotonic medium). Of course, if the erythrocytes are lysed, the remaining cells should then be separated from the lysate, for example by filtration or centrifugation. Of course, regardless of whether the erythrocytes are lysed, the suspended cells can
 25 be washed, re-centrifuged, and resuspended one or more successive times to achieve greater purity. Alternatively, the cells can be separated using a cell sorter or on the basis of cell size and granularity, stem cells being relatively small and agranular. Expression of telomerase can also serve as a stem cell-specific marker. They can also be separated immunohistochemically, for example, by panning or using
 30 magnetic beads. Any of the steps and procedures for isolating the inventive cells can be performed manually, if desired. Alternatively, the process of isolating such cells can be facilitated through a suitable device, many of which are known in the art (see, e.g., U.S. Patent 5,786,207).

Following the final isolation and resuspension, the cells can be cultured and,
 35 if desired, assayed for number and viability to assess the yield. Desirably the cells can be cultured without differentiation using standard cell culture media (e.g., DMEM, typically supplemented with 5-15 % (e.g., 10 %) serum (e.g., fetal bovine

5 Thus, culturing the cells of the present invention without inducing differentiation can be accomplished without specially screened lots of serum, as is generally the case for mesenchymal stem cells (e.g., derived from marrow). Methods for measuring viability and yield are known in the art (e.g., trypan blue exclusion).

Such cells and populations can be clonally expanded, if desired, using a suitable method for cloning cell populations. For example, a proliferated population of cells can be physically picked and seeded into a separate plate (or the well of a multi-well plate). Alternatively, the cells can be subcloned onto a multi-well plate at a statistical ratio for facilitating placing a single cell into each well (e.g., from about 0.1 to about 1 cell/well or even about 0.25 to about 0.5 cells/well, such as 0.5 cells/well). Of course, the cells can be cloned by plating them at low density (e.g., in a petri-dish or other suitable substrate) and isolating them from other cells using devices such as a cloning rings. Alternatively, where an irradiation source is available, clones can be obtained by permitting the cells to grow into a monolayer and then shielding one and irradiating the rest of cells within the monolayer. The surviving cell then will grow into a clonal population. While production of a clonal population can be expanded in any suitable culture medium, a preferred culture condition for cloning stem cells (such as the inventive stem cells or other stem cells) is about $\frac{1}{3}$ F₁₂ medium + 20 % serum (preferably fetal bovine serum) and about $\frac{1}{2}$ standard medium that has been conditioned with stromal cells (e.g., cells from the stromal vascular fraction of liposuction aspirate), the relative proportions being determined volumetrically).

35 In any event, whether clonal or not, the isolated cells can be cultured to a suitable point when their developmental phenotype can be assessed. As mentioned, the inventive cells have at least two of the aforementioned developmental

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cell to conditions that mimic those within fetuses and embryos. For example, the inventive cells or populations can be co-cultured with cells isolated from fetuses or embryos, or in the presence of fetal serum. Along these lines, the cells can be induced to differentiate into any of the aforementioned mesodermal lineages by co-culturing them with mature cells of the respective type, or precursors thereof. Thus, for example, myogenic differentiation can be induced by culturing the inventive cells with myocytes or precursors, and similar results can be achieved with respect to the other tissue types mentioned herein. Other methods of inducing differentiation are known in the art, and many of them can be employed, as appropriate.

After culturing the cells in the differentiating-inducing medium for a suitable time (e.g., several days to a week or more), the cells can be assayed to determine whether, in fact, they have differentiated to acquire physical qualities of a given type of cell. One measurement of differentiation *per se* is telomere length, undifferentiated stem cells having longer telomeres than differentiated cells; thus the cells can be assayed for the level of telomerase activity. Alternatively, RNA or proteins can be extracted from the cells and assayed (via Northern hybridization, rtPCR, Western blot analysis, etc.) for the presence of markers indicative of the desired phenotype. Of course, the cells can be assayed immunohistochemically or stained, using tissue-specific stains. Thus, for example, to assess adipogenic differentiation, the cells can be stained with fat-specific stains (e.g., oil red O, safranin red, sudan black, etc.) or probed to assess the presence of adipose-related factors (e.g., type IV collagen, PPAR- γ , adipin, lipoprotein lipase, etc.). Similarly, osteogenesis can be assessed by staining the cells with bone-specific stains (e.g., alkaline phosphatase, von Kossa, etc.) or probed for the presence of bone-specific markers (e.g., osteocalcin, osteonectin, osteopontin, type I collagen, bone morphogenic proteins, cbfa, etc.). Myogenesis can be assessed by identifying classical morphologic changes (e.g., polynucleated cells, syncytia formation, etc.), or assessed biochemically for the presence of muscle-specific factors (e.g., myo D, myosin heavy chain, NCAM, etc.). Chondrogenesis can be determined by staining the cells using cartilage-specific stains (e.g., alcian blue) or probing the cells for the expression/production of cartilage-specific molecules (e.g., sulfated glycosaminoglycans and proteoglycans (e.g., keratin, chondroitin, etc.) in the medium, type II collagen, etc.). Other methods of assessing developmental phenotype are known in the art, and any of them is appropriate. For example, the cells can be sorted by size and granularity. Also, the cells can be used to generate monoclonal antibodies, which can then be employed to assess whether they

preferentially bind to a given cell type. Correlation of antigenicity can confirm that the stem cell has differentiated along a given developmental pathway.

While the cell can be solitary and isolated from other cells, preferably it is within a population of cells, and the invention provides a defined population including the inventive cell. In some embodiments, the population is heterogeneous. Thus, for example, the population can include support cells for supplying factors to the inventive cells. Of course, the inventive stem cells can themselves serve as support cells for culturing other types of cells (such as other types of stem cells, e.g., as neural stem cells (NSC), hematopoietic stem cells (HPC, particularly CD34⁺ stem cells), embryonic stem cells (ESC) and mixtures thereof), and the population can include such cells. In other embodiments, the population is substantially homogeneous, consisting essentially of the inventive lipo-derived stem cells.

As the inventive cells can be cloned, a substantially homogeneous population containing them can be clonal. Indeed, the invention also pertains to any defined clonal cell population consisting essentially of mesodermal stem cells, connective tissue stem cell, or mixtures thereof. In this embodiment, the cells can be lipo-derived or derived from other mesodermal or connective cell tissues (e.g., bone marrow, muscle, etc.) using methods known in the art. After the isolation, the cells can be expanded clonally as described herein.

The inventive cells (and cell populations) can be employed for a variety of purposes. As mentioned, the cells can support the growth and expansion of other cell types, and the invention pertains to methods for accomplishing this. In one aspect, the invention pertains to a method of conditioning culture medium using the inventive stem cells and to conditioned medium produced by such a method. The medium becomes conditioned upon exposing a desired culture medium to the cells under conditions sufficient for the cells to condition it. Typically, the medium is used to support the growth of the inventive cells, which secrete hormones, cell matrix material, and other factors into the medium. After a suitable period (e.g., one or a few days), the culture medium containing the secreted factors can be separated from the cells and stored for future use. Of course, the inventive cells and populations can be re-used successively to condition medium, as desired. In other applications (e.g., for co-culturing the inventive cells with other cell types), the cells can remain within the conditioned medium. Thus, the invention provides a conditioned medium obtained using this method, which either can contain the inventive cells or be substantially free of the inventive cells, as desired.

The conditioned medium can be used to support the growth and expansion of desired cell types, and the invention provides a method of culturing cells (particularly

The cell can be maintained under any suitable condition for culturing them, such as are known in the art. Desirably, the method permits successive rounds of mitotic

division of the cell to form an expanded population. The exact conditions (e.g., temperature, CO₂ levels, agitation, presence of antibiotics, etc.) will depend on the

other constituents of the medium and on the cell type. However, optimizing these parameters are within the ordinary skill in the art. In some embodiments, it is

desirable for the medium to be substantially free of the lipo-derived cells employed to condition the medium as described herein. However, in other embodiments, it is

desirable for the lipo-derived cells to remain in the conditioned medium and co-cultured with the cells of interest. Indeed, as the inventive lipo-derived cells can

express cell-surface mediators of intercellular communication, it often is desirable the inventive cells and the desired other cells to be co-cultured under conditions in

which the two cell types are in contact. This can be achieved, for example, by seeding the cells as a heterogeneous population of cells onto a suitable culture

substrate. Alternatively, the inventive lipo-derived cells can first be grown to confluence, which will serve as a substrate for the second desired cells to be cultured.

within the conditioned medium.

In another embodiment, the inventive lipo-derived cells can be genetically

gene transfer vector comprising a nucleic acid including a transgene, such that the nucleic acid is introduced into the cell under conditions appropriate for the transgene to be expressed within the cell. The transgene generally is an expression cassette

coding polynucleotide can encode a protein, or it can encode biologically active RNA (e.g., antisense RNA or a ribozyme). Thus, for example, the coding polynucleotide

can encode a gene conferring resistance to a toxin, a hormone (such as peptide growth hormones, hormone releasing factors, sex hormones, adrenocorticotrophic

hormones, cytokines (e.g., interferins, interleukins, lymphokines), etc.), a cell-surface-bound intracellular signaling moiety (e.g., cell adhesion molecules, hormo-

receptors, etc.), a factor promoting a given lineage of differentiation, etc. Of course, where it is desired to employ gene transfer technology to deliver a given transgene

its sequence will be known.

Within the expression cassette, the coding polynucleotide is operably linked to a suitable promoter. Examples of suitable promoters include prokaryotic promoters and viral promoters (e.g., retroviral ITRs, LTRs, immediate early viral promoters (IEp), such as herpesvirus IEp (e.g., ICP4-IEp and ICP0-IEp),
 5 cytomegalovirus (CMV) IEp, and other viral promoters, such as Rous Sarcoma Virus (RSV) promoters, and Murine Leukemia Virus (MLV) promoters). Other suitable promoters are eukaryotic promoters, such as enhancers (e.g., the rabbit β -globin regulatory elements), constitutively active promoters (e.g., the β -actin promoter, etc.), signal specific promoters (e.g., inducible promoters such as a promoter responsive to
 10 RU486, etc.), and tissue-specific promoters. It is well within the skill of the art to select a promoter suitable for driving gene expression in a predefined cellular context. The expression cassette can include more than one coding polynucleotide, and it can include other elements (e.g., polyadenylation sequences, sequences encoding a membrane-insertion signal or a secretion leader, ribosome entry
 15 sequences, transcriptional regulatory elements (e.g., enhancers, silencers, etc.), and the like), as desired.

The expression cassette containing the transgene should be incorporated into a genetic vector suitable for delivering the transgene to the cells. Depending on the desired end application, any such vector can be so employed to genetically modify
 20 the cells (e.g., plasmids, naked DNA, viruses such as adenovirus, adeno-associated virus, herpesviruses, lentiviruses, papillomaviruses, retroviruses, etc.). Any method of constructing the desired expression cassette within such vectors can be employed, many of which are well known in the art (e.g., direct cloning, homologous recombination, etc.). Of course, the choice of vector will largely determine the
 25 method used to introduce the vector into the cells (e.g., by protoplast fusion, calcium-phosphate precipitation, gene gun, electroporation, infection with viral vectors, etc.), which are generally known in the art.

The genetically altered cells can be employed as bioreactors for producing the product of the transgene. In other embodiments, the genetically modified cells are
 30 employed to deliver the transgene and its product to an animal. For example, the cells, once genetically modified, can be introduced into the animal under conditions sufficient for the transgene to be expressed *in vivo*.

In addition to serving as useful targets for genetic modification, many cells and populations of the present invention secrete hormones (e.g., cytokines, peptide or
 35 other (e.g., monobutyrin) growth factors, etc.). Some of the cells naturally secrete such hormones upon initial isolation, and other cells can be genetically modified to secrete hormones, as discussed herein. The cells of the present invention that secrete

hormones can be used in a variety of contexts *in vivo* and *in vitro*. For example, such cells can be employed as bioreactors to provide a ready source of a given hormone, and the invention pertains to a method of obtaining hormones from such cells. In accordance with the method, the cells are cultured, under suitable conditions for them to secrete the hormone into the culture medium. After a suitable period of time, and preferably periodically, the medium is harvested and processed to isolate the hormone from the medium. Any standard method (e.g., gel or affinity chromatography, dialysis, lyophilization, etc.) can be used to purify the hormone from the medium, many of which are known in the art.

In other embodiments, cells (and populations) of the present invention secreting hormones can be employed as therapeutic agents. Generally, such methods involve transferring the cells to desired tissue, either *in vitro* (e.g., as a graft prior to implantation or engrafting) or *in vivo*, to animal tissue directly. The cells can be transferred to the desired tissue by any method appropriate, which generally will vary according to the tissue type. For example, cells can be transferred to a graft by bathing the graft (or infusing it) with culture medium containing the cells. Alternatively, the cells can be seeded onto the desired site within the tissue to establish a population. Cells can be transferred to sites *in vivo* using devices such as catheters, trocars, cannulae, stents (which can be seeded with the cells), etc. For these applications, preferably the cell secretes a cytokine or growth hormone such as human growth factor, fibroblast growth factor, nerve growth factor, insulin-like growth factors, hemopoietic stem cell growth factors, members of the fibroblast growth factor family, members of the platelet-derived growth factor family, vascular and endothelial cell growth factors, members of the TGF β family (including bone morphogenic factor), or enzymes specific for congenital disorders (e.g., dystrophin).

In one application, the invention provides a method of promoting the closure of a wound within a patient using such cells. In accordance with the method, the inventive cells secreting the hormone are transferred to the vicinity of a wound under conditions sufficient for the cell to produce the hormone. The presence of the hormone in the vicinity of the wound promotes closure of the wound. The method promotes closure of both external (e.g., surface) and internal wounds. Wounds to which the present inventive method is useful in promoting closure include, but are not limited to, abrasions, avulsions, blowing wounds, burn wounds, contusions, gunshot wounds, incised wounds, open wounds, penetrating wounds, perforating wounds, puncture wounds, seton wounds, stab wounds, surgical wounds, subcutaneous wounds, or tangential wounds. The method need not achieve complete healing or closure of the wound; it is sufficient for the method to promote any degree

of wound closure. In this respect, the method can be employed alone or as an adjunct to other methods for healing wounded tissue.

Where the inventive cells secrete an angiogenic hormone (e.g., vascular growth factor, vascular and endothelial cell growth factor, etc.), they (as well as populations containing them) can be employed to induce angiogenesis within tissues. Thus, the invention provides a method of promoting neovascularization within tissue using such cells. In accordance with this method, the cell is introduced the desired tissue under conditions sufficient for the cell to produce the angiogenic hormone. The presence of the hormone within the tissue promotes neovascularization within the tissue.

Because the inventive stem cells have a developmental phenotype, they can be employed in tissue engineering. In this regard, the invention provides a method of producing animal matter comprising maintaining the inventive cells under conditions sufficient for them to expand and differentiate to form the desired matter. The matter can include mature tissues, or even whole organs, including tissue types into which the inventive cells can differentiate (as set forth herein). Typically, such matter will comprise adipose, cartilage, heart, dermal connective tissue, blood tissue, muscle, kidney, bone, pleural, splanchnic tissues, vascular tissues, and the like. More typically, the matter will comprise combinations of these tissue types (i.e., more than one tissue type). For example, the matter can comprise all or a portion of an animal organ (e.g., a heart, a kidney) or a limb (e.g., a leg, a wing, an arm, a hand, a foot, etc.). Of course, in as much as the cells can divide and differentiate to produce such structures, they can also form anlagen of such structures. At early stages, such anlagen can be cryopreserved for future generation of the desired mature structure or organ.

To produce such structures, the inventive cells and populations are maintained under conditions suitable for them to expand and divide to form the desired structures. In some applications, this is accomplished by transferring them to an animal (i.e., *in vivo*) typically at a sight at which the new matter is desired. Thus, for example, the invention can facilitate the regeneration of tissues (e.g., bone, muscle, cartilage, tendons, adipose, etc.) within an animal where the cells are implanted into such tissues. In other embodiments, and particularly to create anlagen, the cells can be induced to differentiate and expand into tissues *in vitro*. In such applications, the cells are cultured on substrates that facilitate formation into three-dimensional structures conducive for tissue development. Thus, for example, the cells can be cultured or seeded onto a bio-compatible lattice, such as one that includes extracellular matrix material, synthetic polymers, cytokines, growth factors,

etc. Such a lattice can be molded into desired shapes for facilitating the development of tissue types. Also, at least at an early stage during such culturing, the medium and/or substrate is supplemented with factors (e.g., growth factors, cytokines, extracellular matrix material, etc.) that facilitate the development of appropriate tissue types and structures. Indeed, in some embodiments, it is desired to co-culture the cells with mature cells of the respective tissue type, or precursors thereof, or to expose the cells to the respective conditioned medium, as discussed herein.

To facilitate the use of the inventive lipo-derived cells and populations for producing such animal matter and tissues, the invention provides a composition including the inventive cells (and populations) and biologically compatible lattice. Typically, the lattice is formed from polymeric material, having fibers as a mesh or sponge, typically with spaces on the order of between about 100 μm and about 300 μm . Such a structure provides sufficient area on which the cells can grow and proliferate. Desirably, the lattice is biodegradable over time, so that it will be absorbed into the animal matter as it develops. Suitable polymeric lattices, thus, can be formed from monomers such as glycolic acid, lactic acid, propyl fumarate, caprolactone, hyaluronan, hyaluronic acid, and the like. Other lattices can include proteins, polysaccharides, polyhydroxy acids, polyorthoesters, polyanhydrides, polyphosphazenes, or synthetic polymers (particularly biodegradable polymers). Of course, a suitable polymer for forming such lattice can include more than one monomers (e.g., combinations of the indicated monomers). Also, the lattice can also include hormones, such as growth factors, cytokines, and morphogens (e.g., retinoic acid, aradonic acid, etc.), desired extracellular matrix molecules (e.g., fibronectin, laminin, collagen, etc.), or other materials (e.g., DNA, viruses, other cell types, etc.) as desired.

To form the composition, the cells are introduced into the lattice such that they permeate into the interstitial spaces therein. For example, the matrix can be soaked in a solution or suspension containing the cells, or they can be infused or injected into the matrix. A particularly preferred composition is a hydrogel formed by crosslinking of a suspension including the polymer and also having the inventive cells dispersed therein. This method of formation permits the cells to be dispersed throughout the lattice, facilitating more even permeation of the lattice with the cells. Of course, the composition also can include mature cells of a desired phenotype or precursors thereof, particularly to potentate the induction of the inventive stem cells to differentiate appropriately within the lattice (e.g., as an effect of co-culturing such cells within the lattice).

The composition can be employed in any suitable manner to facilitate the growth and generation of the desired tissue types, structures, or anlagen. For example, the composition can be constructed using three-dimensional or sterotactic modeling techniques. Thus, for example, a layer or domain within the composition

5 can be populated by cells primed for osteogenic differentiation, and another layer or domain within the composition can be populated with cells primed for myogenic and/or chondrogenic development. Bringing such domains into juxtaposition with each other facilitates the molding and differentiation of complex structures including multiple tissue types (e.g., bone surrounded by muscle, such as found in a limb). To

10 direct the growth and differentiation of the desired structure, the composition can be cultured *ex vivo* in a bioreactor or incubator, as appropriate. In other embodiments, the structure is implanted within the host animal directly at the site in which it is desired to grow the tissue or structure. In still another embodiment, the composition can be grafted on a host (typically an animal such as a pig, baboon, etc.), where it

15 will grow and mature until ready for use. Thereafter, the mature structure (or anlage) is excised from the host and implanted into the host, as appropriate.

Lattices suitable for inclusion into the composition can be derived from any suitable source (e.g., matrigel), and some commercial sources for suitable lattices exist (e.g., suitable of polyglycolic acid can be obtained from sources such as

20 Ethicon, N.J.). Another suitable lattice can be derived from the acellular portion of adipose tissue – i.e., adipose tissue extracellular matrix matter substantially devoid of cells, and the invention provides such a lipo-derived lattice. Typically, such lipo-derived lattice includes proteins such as proteoglycans, glycoproteins, hyaluronins, fibronectins, collagens (type I, type II, type III, type IV, type V, type VI, etc.), and

25 the like, which serve as excellent substrates for cell growth. Additionally, such lipo-derived lattices can include hormones, preferably cytokines and growth factors, for facilitating the growth of cells seeded into the matrix.

The lipo-derived matrix can be isolated from adipose tissue similarly as described above, except that it will be present in the acellular fraction. For example,

30 adipose tissue or derivatives thereof (e.g., a fraction of the cells following the centrifugation as discussed above) can be subjected to sonic or thermal energy and/or enzymatic processed to recover the matrix material. Also, desirably the cellular fraction of the adipose tissue is disrupted, for example by treating it with lipases, detergents, proteases, and/or by mechanical or sonic disruption (e.g., using a

35 homogenizer or sonicator). However isolated, the material is initially identified as a viscous material, but it can be subsequently treated, as desired, depending on the desired end use. For example, the raw matrix material can be treated (e.g., dialyzed

or treated with proteases or acids, etc.) to produce a desirable lattice material. Thus the lattice can be prepared in a hydrated form or it can be dried or lyophilized into a substantially anhydrous form or a powder. Thereafter, the powder can be rehydrated for use as a cell culture substrate, for example by suspending it in a suitable cell culture medium. In this regard, the lipo-derived lattice can be mixed with other suitable lattice materials, such as described above. Of course, the invention pertains to compositions including the lipo-derived lattice and cells or populations of cells, such as the inventive lipo-derived cells and other cells as well (particularly other types of stem cells).

As discussed above, the cells, populations, lattices, and compositions of the invention can be used in tissue engineering and regeneration. Thus, the invention pertains to an implantable structure (i.e., an implant) incorporating any of these inventive features. The exact nature of the implant will vary according to the use to which it is to be put. The implant can be or comprise, as described, mature tissue, or it can include immature tissue or the lattice. Thus, for example, one type of implant can be a bone implant, comprising a population of the inventive cells that are undergoing (or are primed for) osteogenic differentiation, optionally seeded within a lattice of a suitable size and dimension, as described above. Such an implant can be injected or engrafted within a host to encourage the generation or regeneration of mature bone tissue within the patient. Similar implants can be used to encourage the growth or regeneration of muscle, fat, cartilage, tendons, etc., within patients. Other types of implants are anlagen (such as described herein), e.g., limb buds, digit buds, developing kidneys, etc, that, once engrafted onto a patient, will mature into the appropriate structures.

The lipo-derived lattice can conveniently be employed as part of a cell culture kit. Accordingly, the invention provides a kit including the inventive lipo-derived lattice and one or more other components, such as hydrating agents (e.g., water, physiologically-compatible saline solutions, prepared cell culture media, serum or derivatives thereof, etc.), cell culture substrates (e.g., culture dishes, plates, vials, etc.), cell culture media (whether in liquid or powdered form), antibiotic compounds, hormones, and the like. While the kit can include any such ingredients, preferably it includes all ingredients necessary to support the culture and growth of desired cell types upon proper combination. Of course, if desired, the kit also can include cells (typically frozen), which can be seeded into the lattice as described herein.

While many aspects of the invention pertain to tissue growth and differentiation, the invention has other applications as well. For example, the lipo-derived lattice can be used as an experimental reagent, such as in developing

- improved lattices and substrates for tissue growth and differentiation. The lipo-derived lattice also can be employed cosmetically, for example, to hide wrinkles, scars, cutaneous depressions, etc., or for tissue augmentation. For such applications, preferably the lattice is stylized and packaged in unit dosage form. If desired, it can be admixed with carriers (e.g., solvents such as glycerine or alcohols), perfumes, antibiotics, colorants, and other ingredients commonly employed in cosmetic products. The substrate also can be employed autologously or as an allograft, and it can be used as, or included within, ointments or dressings for facilitating wound healing. The lipo-derived cells can also be used as experimental reagents. For example, they can be employed to help discover agents responsible for early events in differentiation. For example, the inventive cells can be exposed to a medium for inducing a particular line of differentiation and then assayed for differential expression of genes (e.g., by random-primed PCR or electrophoresis or protein or RNA, etc.).
- As any of the steps for isolating the inventive stem cells or the lipo-derived lattice, the invention provides a kit for isolating such reagents from adipose tissues. The kit can include a means for isolating adipose tissue from a patient (e.g., a cannula, a needle, an aspirator, etc.), as well as a means for separating stromal cells (e.g., through methods described herein). The kit can be employed, for example, as a bedside source of stem cells that can then be re-introduced from the same individual as appropriate. Thus, the kit can facilitate the isolation of lipo-derived stem cells for implantation in a patient needing regrowth of a desired tissue type, even in the same procedure. In this respect, the kit can also include a medium for differentiating the cells, such as those set forth herein. As appropriate, the cells can be exposed to the medium to prime them for differentiation within the patient as needed. Of course, the kit can be used as a convenient source of stem cells for *in vitro* manipulation (e.g., cloning or differentiating as described herein). In another embodiment, the kit can be employed for isolating a lipo-derived lattice as described herein.

EXAMPLES

While one of skill in the art is fully able to practice the instant invention upon reading the foregoing detailed description, the following examples will help elucidate some of its features. In particular, they demonstrate the isolation of a human lipo-
5 derived stem cell substantially free of mature adipocytes, the isolation of a clonal population of such cells, the ability of such cells to differentiate *in vivo* and *in vitro*, and the capacity of such cells to support the growth of other types of stem cells. The examples also demonstrate the isolation of a lipo-derived lattice substantially free of cells that is capable of serving as a suitable substrate for cell culture. Of course, as
10 these examples are presented for purely illustrative purposes, they should not be used to construe the scope of the invention in a limited manner, but rather should be seen as expanding upon the foregoing description of the invention as a whole.

The procedures employed in these examples, such as surgery, cell culture, enzymatic digestion, histology, and molecular analysis of proteins and
15 polynucleotides, are familiar to those of ordinary skill in this art. As such, and in the interest of brevity, experimental details are not recited in detail.

EXAMPLE 1

This example demonstrates the isolation of a human lipo-derived stem cell
20 substantially free of mature adipocytes.

Raw liposuction aspirate was obtained from patients undergoing elective surgery. Prior to the liposuction procedures, the patients were given epinephrine to minimize contamination of the aspirate with blood. The aspirate was strained to separate associated adipose tissue pieces from associated liquid waste. Isolated tissue
25 was rinsed thoroughly with neutral phosphate buffered saline and then enzymatically dissociated with 0.075 % w/v collagenase at 37 °C for about 20 minutes under intermittent agitation. Following the digestion, the collagenase was neutralized, and the slurry was centrifuged at about 260g for about 10 minutes, which produced a multi-layered supernatant and a cellular pellet. The supernatant was removed and
30 retained for further use, and the pellet was resuspended in an erythrocyte-lysing solution and incubated without agitation at about 25 °C for about 10 minutes. Following incubation, the medium was neutralized, and the cells were again centrifuged at about 250g for about 10 minutes. Following the second centrifugation, the cells were resuspended, and assessed for viability (using trypan blue exclusion) and
35 cell number. Thereafter, they were plated at a density of about 1×10^6 cells/100 mm dish. They were cultured at 37 °C in DMEM + fetal bovine serum (about 10 %) in about 5 % CO₂.

The majority of the cells were adherent, small, mononucleic, relatively agranular, fibroblast-like cells containing no visible lipid droplets. The majority the cells stained negatively with oil-red O and von Kossa. The cells were also assayed for expression of telomerase (using a commercially available TRAP assay kit), using HeLa cells and HN-12 cells as positive controls. Human foreskin fibroblasts and HN-12 heated cell extracts were used as negative controls. Telomeric products were resolved onto a 12.5% polyacrylamide cells and the signals determined by phosphorimaging. Telemeric ladders representing telomerase activity were observed in the adipose-derived stem cells as well as the positive controls. No ladders were observed in the negative controls.

Thus, these cells were not identifiable as myocytes, adipocytes, chondrocytes, osteocytes, or blood cells. These results demonstrate that the adipose-derived cells express telomerase activity similar to that previously reported for human stem cells.

Subpopulations of these cells were then exposed to the following media to assess their developmental phenotype:

Adipogenesis	Osteogenesis	Myogenesis	Chondrogenesis
DMEM 10 % FBS 0.5 mM IBMX 1 μ M dexamethasone 10 μ M insulin 200 μ M indomethacin 1 % ABAM	DMEM 10 % FBS 5 % horse serum 0.1 μ M dexamethasone 50 μ M ascorbate-2-phosphate 10 mM β -glycerophosphate 1% ABAM	DMEM 10 % FBS 5 % horse serum 50 μ M hydrocortisone 1 % ABAM	DMEM 1 % FBS 6.25 μ g/ml insulin 6.25 μ g/ml transferrin 10 ng/ml TGF β 1 50 nM ascorbate-2-phosphate 1 % ABAM

A population was cultured at high density in the chondrogenic medium for several weeks. Histological analysis of the tissue culture and paraffin sections was performed with H&E, alcian blue, toluidine blue, and Goldner's trichrome staining at 2, 7, and 14 days. Immunohistochemistry was performed using antibodies against chondroitin-4-sulfate and keratin sulfate and type II collagen. Qualitative estimate of matrix staining was also performed. The results indicated that cartilaginous spheroid nodules with a distinct border of perichondral cells formed as early as 48 hours after initial treatment. Untreated control cells exhibited no evidence of chondrogenic differentiation. These results confirm that the stem cells have chondrogenic developmental phenotype.

A population was cultured until near confluence and then exposed to the adipogenic medium for several weeks. The population was examined at two and four weeks after plating by colorimetric assessment of relative opacity following oil red-O

staining. Adipogenesis was determined to be underway at two weeks and quite advanced at four weeks (relative opacity of 1 and 5.3, respectively). Bone marrow-derived stem cells were employed as a positive control, and these cells exhibited slightly less adipogenic potential (relative density of 0.7 and 2.8, respectively).

5 A population was cultured until near confluence and then exposed to the osteogenic medium for several weeks. The population was examined at two and four weeks after plating by colorimetric assessment of relative opacity following von Kossa staining. Osteogenesis was determined to be underway at two weeks and quite
10 advanced at four weeks (relative opacity of 1.1 and 7.3, respectively). Bone marrow-derived stem cells were employed as a positive control, and these cells exhibited slightly less osteogenic potential (relative density of 0.2 and 6.6, respectively).

A population was cultured until near confluence and then exposed to the myogenic medium for several weeks. The population was examined at one, three, and six weeks after plating by assessment of multinucleated cells and expression of muscle-specific proteins (MyoD and myosin heavy chain). Human foreskin fibroblasts and
15 skeletal myoblasts were used as controls. Cells expressing MyoD and myosin were found at all time points following exposure to the myogenic medium in the stem cell population, and the proportion of such cells increased at 3 and 6 weeks. Multinucleated cells were observed at 6 weeks. In contrast, the fibroblasts exhibited
20 none of these characteristics at any time points.

These results demonstrate the isolation of a human lipo-derived pluripotent stem cell substantially free of mature adipocytes.

EXAMPLE 2

25 This example demonstrates that lipo-derived stem cells do not differentiate in response to 5-azacytidine.

Lipo-derived stem cells obtained in accordance with Example 1 were cultured in the presence of 5-azacytidine. In contrast with bone marrow-derived stem cells, exposure to this agent did not induce myogenic differentiation (see Wakitani et al.,
30 *supra*).

EXAMPLE 3

This example demonstrates the generation of a clonal population of human lipo-derived stem cells.

35 Cells isolated in accordance with the procedure set forth in Example 1 were plated at about 5,000 cells/100 mm dish and cultured for a few days as indicated in Example 1. After some rounds of cell division, some clones were picked with a

cloning ring and transferred to wells in a 48 well plate. These cells were cultured for several weeks, changing the medium twice weekly, until they were about 80 % to about 90 % confluent (at 37 °C in about 5% CO₂ in 1/3 F₁₂ medium + 20 % fetal bovine serum and 1/3 standard medium that was first conditioned by the cells isolated in Example 1, "cloning medium"). Thereafter, each culture was transferred to a 35 mm dish and grown, and then retransferred to a 100 mm dish and grown until close to confluent. Following this, one cell population was frozen, and the remaining populations were plated on 12 well plates, at 1000 cells/well.

The cells were cultured for more than 15 passages in cloning medium and monitored for differentiation as indicated in Example 1. The undifferentiated state of each clone remained true after successive rounds of differentiation.

Populations of the clones then were established and exposed to adipogenic, chondrogenic, myogenic, and osteogenic medium as discussed in Example 1. It was observed that at least one of the clones was able to differentiate into bone, fat, cartilage, and muscle when exposed to the respective media, and most of the clones were able to differentiate into at least three types of tissues. The capacity of the cells to develop into muscle and cartilage further demonstrates the pluripotentiality of these lipo-derived stem cells.

These results demonstrate that the lipo-derived stem cells can be maintained in an undifferentiated state for many passages without the requirement for specially pre-screened lots of serum. The results also demonstrate that the cells retain pluripotentiality following such extensive passaging, proving that the cells are indeed stem cells and not merely committed progenitor cells.

EXAMPLE 4

This example demonstrates the lipo-derived stem cells can support the culture of other types of stem cells.

Human lipo-derived stem cells were passaged onto 96 well plates at a density of about 30,000/well, cultured for one week and then irradiated. Human CD34⁺ hematopoietic stem cells isolated from umbilical cord blood were then seeded into the wells. Co-cultures were maintained in MyeloCult H5100 media, and cell viability and proliferation were monitored subjectively by microscopic observation. After two weeks of co-culture, the hematopoietic stem cells were evaluated for CD34 expression by flow cytometry.

Over a two-week period of co-culture with stromal cells, the hematopoietic stem cells formed large colonies of rounded cells. Flow analysis revealed that 62% of the cells remained CD34⁺. Based on microscopic observations, human adipo-derived

stromal cells maintained the survival and supported the growth of human hematopoietic stem cells derived from umbilical cord blood.

These results demonstrate that stromal cells from human subcutaneous adipose tissue are able to support the *ex vivo* maintenance, growth and differentiation of other stem cells.

EXAMPLE 5

This example demonstrates that the lipo-derived stem cells can differentiate *in vivo*.

Four groups (A-D) of 12 athymic mice each were implanted subcutaneously with hydroxyapatite/tricalcium phosphate cubes containing the following: Group A contained lipo-derived stem cells that had been pretreated with osteogenic medium as set forth in Example 1. Group B contained untreated lipo-derived stem cells. Group C contained osteogenic medium but no cells. Group D contained non-osteogenic medium and no cells. Within each group, six mice were sacrificed at three weeks, and the remaining mice sacrificed at eight weeks following implantation. The cubes were extracted, fixed, decalcified, and sectioned. Each section was analyzed by staining with H&E, Mallory bone stain, and immunostaining for osteocalcin.

Distinct regions of osteoid-like tissue staining for osteocalcin and Mallory bone staining was observed in sections from groups A and B. Substantially more osteoid tissue was observed in groups A and B than in the other groups ($p < 0.05$ ANOVA), but no significant difference in osteogenesis was observed between groups A and B. Moreover, a qualitative increase in bone growth was noted in both groups A and B between 3 and 8 weeks. These results demonstrate that the lipo-derived stem cells can differentiate *in vivo*.

EXAMPLE 6

This example demonstrates the isolation of a lipo-derived lattice substantially devoid of cells.

In one protocol withheld supernatant from Example 1 was subjected to enzymatic digestion for three days in 0.05 % trypsin EDTA/ 100 U/ml deoxyribonuclease to destroy the cells. Every day the debris was rinsed in saline and fresh enzyme was added. Thereafter the material was rinsed in saline and resuspended in 0.05 % collagenase and about 0.1% lipase to partially digest the proteins and fat present. This incubation continued for two days.

In another protocol, the withheld supernatant from Example 1 was incubated in EDTA to eliminate any epithelial cells. The remaining cells were lysed using a buffer

After both preparatory protocols, remaining substance was washed and identified as a gelatinous mass. Microscopic analysis of this material revealed that it contained no cells, and it was composed of high amounts of collagen (likely type IV) and a wide variety of growth factors. Preparations of this material have supported the growth of cells, demonstrating it to be an excellent substrate for tissue culture.

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As regards the claims in particular, the term “consisting essentially of” indicates that unlisted ingredients or steps that do not materially affect the basic and novel properties of the invention can be employed in addition to the specifically recited ingredients or steps. In contrast, terms such as “comprising,” “having,” and “including” indicate that any ingredients or steps can be present in addition to those recited. The term “consisting of” indicates that only the recited ingredients or steps are present, but does not foreclose the possibility that equivalents of the ingredients or steps can substitute for those specifically recited.

WHAT IS CLAIMED IS:

1. A mammalian lipo-derived stem cell substantially free of mature adipocytes.
2. The cell of claim 1, which can be cultured in DMEM + about 10% fetal bovine serum for at least 15 passages without differentiating.
3. The cell of claim 2, which has two or more developmental phenotypes selected from the group of developmental phenotypes consisting of adipogenic, chondrogenic, cardiogenic, dermatogenic, hematopoietic, hemangiogenic, myogenic, nephrogenic, neurogenic, neuralgiagenic, urogenitogenic, osteogenic, pericardiogenic, peritoneogenic, pleurogenic, splanchnogenic, and stromal developmental phenotypes.
4. The cell of any of claims 1-3, which is human.
5. The cell of any of claims 1-4, which is genetically modified.
6. The cell of any of claims 1-5, which has a cell-surface bound intercellular signaling moiety.
7. The cell of any of claims 1-5, which secretes a hormone.
8. The cell of claim 7, wherein the hormone is selected from the group of hormones consisting of cytokines and growth factors.
9. A defined cell population comprising a cell of any of claims 1-8.
10. The defined cell population of claim 9, which is heterogeneous.
11. The defined cell population of claim 9 or 10, further compressing a stem cell selected from the group of cells consisting of neural stem cells (NSC), hematopoietic stem cells (HPC), embryonic stem cells (ESC) and mixtures thereof.
12. The defined cell population of claim 9, which consists essentially of cells according to any of claims 1-8.
13. The defined cell population of claim 9 or 12, which is substantially homogenous.
14. The defined cell population of claim 13, which is clonal.
15. A defined cell population consisting essentially of mesodermal stem cells (MHC), connective tissue stem cell (CTSC), or mixtures thereof, wherein the population is clonal.
16. The population of claim 15, wherein the stem cells have two or more developmental phenotypes selected from the group of developmental phenotypes consisting of adipogenic, chondrogenic, cardiogenic, dermatogenic, hematopoietic, hemangiogenic, myogenic, nephrogenic, neurogenic, neuralgiagenic, urogenitogenic, osteogenic, pericardiogenic, peritoneogenic, pleurogenic, splanchnogenic, and stromal developmental phenotypes.

17. A lipo-derived lattice comprising adipose tissue extracellular matrix matter substantially devoid of cells.

18. The lipo-derived lattice of claim 17, comprising a human protein, proteoglycan, glycoprotein, hyaluronin, or fibronectin molecule.

5 19. The lipo-derived lattice of claim 17 or 18, comprising a collagen selected from the group of collagens consisting of type I, type II, type III, type IV, type V, type VI collagen.

20. The lipo-derived lattice of any of claims 17-19, comprising a hormone.

10 21. The lipo-derived lattice of claim 20, wherein the hormone is selected from the group of hormones consisting of cytokines and growth factors.

22. The lipo-derived lattice of any of claims 17-21, which is substantially anhydrous.

23. The lipo-derived lattice of any of claims 17-22, which is lyophilized.

24. The lipo-derived lattice of any of claims 17-21, which is hydrated.

15 25. A kit comprising the lipo-derived lattice of any of claims 17-24 and one or more components selected from the group of components consisting of hydrating agents, cell culture substrates, cell culture media, antibiotic compounds, and hormones.

26. A composition comprising a cell and the lipo-derived lattice of any of claims 17-24.

20 27. A composition comprising the cell of any of claims 1-8 and a biologically compatible lattice.

28. A composition comprising the population of any of claims 9-16 and a biologically compatible lattice.

25 29. The composition of claim 27 or 28, wherein the lattice comprises polymeric material.

30. The composition of claim 29, wherein the polymeric material is formed of polymer fibers as a mesh or sponge.

31. The composition of claim 29 or 30, wherein the polymeric material comprises monomers selected from the group of monomers consisting of glycolic acid, lactic acid, propyl fumarate, caprolactone, hyaluronan, hyaluronic acid and combinations thereof.

32. The composition of any of claims 29-31, wherein the polymeric material comprises proteins, polysaccharides, polyhydroxy acids, polyorthoesters, polyanhydrides, polyphosphazenes, synthetic polymers or combinations thereof.

35 33. The composition of any of claims 29-32, wherein the polymeric material is a hydrogel formed by crosslinking of a polymer suspension having the cells dispersed therein.

34. The composition of any of claims 29-33, wherein the lattice further comprises a hormone selected from the group of hormones consisting of cytokines and growth factors.

35. The composition of any of claims 29-34, wherein the lattice is the lipoderived lattice of any of claims 17-24.

36. A method of obtaining a genetically-modified cell comprising exposing the cell of any of claims 1-8 to a gene transfer vector comprising a nucleic acid including a transgene, whereby the nucleic acid is introduced into the cell under conditions whereby the transgene is expressed within the cell.

37. The method of claim 36, wherein the transgene encodes a protein conferring resistance to a toxin.

38. A method of delivering a transgene to an animal comprising (a) obtaining a genetically-modified cell in accordance with claim 36 or 37 and (b) introducing the cell into the animal, such that the transgene is expressed *in vivo*.

39. A method of differentiating the cell of any of claims 1-8 comprising culturing the cell in a morphogenic medium under conditions sufficient for the cell to differentiate.

40. The method of claim 39, wherein the medium is an adipogenic, chondrogenic, cardiogenic, dermatogenic, embryonic, fetal, hematopoietic, hemangiogenic, myogenic, nephrogenic, neurogenic, neuralgiagenic, urogenitogenic, osteogenic, pericardiogenic, peritoneogenic, pleurogenic, and splanchnogenic, or stromogenic media.

41. The method of claim 39 or 40, wherein the morphogenic medium is an adipogenic medium and the cell is monitored to identify adipogenic differentiation.

42. The method of claim 39 or 40, wherein the morphogenic medium is a chondrogenic medium and the cell is monitored to identify chondrogenic differentiation.

43. The method of claim 39 or 40, wherein the morphogenic medium is an embryonic or fetal medium and the cell is monitored to identify embryonic or fetal phenotype.

44. The method of claim 39 or 40, wherein the morphogenic medium is a myogenic medium and the cell is monitored to identify myogenic differentiation.

45. The method of claim 39 or 40, wherein the morphogenic medium is an osteogenic medium and the cell is monitored to identify osteogenic differentiation.

46. The method of claim 39 or 40, wherein the morphogenic medium is a stromal medium and the cell is monitored to identify stromal or hematopoietic differentiation.

47. The method of any of claims 39-46, wherein the cell differentiates *in vitro*.
48. The method of any of claims 39-46, wherein the cell differentiates *in vivo*.
49. A method of producing hormones, comprising (a) culturing the cell of claim 7 or 8 within a medium under conditions sufficient for the cell to secrete the hormone into the medium and (b) isolating the hormone from the medium.
50. A method of promoting the closure of a wound within a patient comprising introducing the cell of claim 7 or 8 into the vicinity of a wound under conditions sufficient for the cell to produce the hormone, whereby the presence of the hormone promotes closure of the wound.
51. A method of promoting neovascularization within tissue, comprising introducing the cell of claim 7 or 8 into the tissue under conditions sufficient for the cell to produce the hormone, whereby the presence of the hormone promotes neovascularization within the tissue.
52. The method of claim 51, wherein the tissue is within an animal.
53. The method of claim 51 or 52, wherein the tissue is a graft.
54. The method of any of claims 49-53, wherein the hormone is a growth factor selected from the group of growth factor consisting of human growth factor, nerve growth factor, vascular and endothelial cell growth factor, and members of the TGF β superfamily.
55. A method of conditioning culture medium comprising exposing a cell culture medium to the cell of any of claims 1-7 under conditions sufficient for the cell to condition the medium.
56. The method of claim 55, wherein the medium is separated from the cell after it has been conditioned.
57. The method of any of claims 36-56, wherein the cell is within a population of any of claims 9-16.
58. A conditioned culture medium produced in accordance with the method of claim 55 or 56.
59. The conditioned culture medium of claim 58, which is substantially free of a cell of any of claims 1-7.
60. A method of culturing a stem cell comprising maintaining a stem cell in the conditioned medium of claim 58 or 59 under conditions for the stem cell to remain viable.
61. The method of claim 60, which further comprises permitting successive rounds of mitotic division of the stem cell to form an expanded population of stem cells.

62. The method of claim 60 or 61, wherein the medium is substantially free of the lipo-derived cells of any of claims 1-7.

63. The method of any of claims 60-62, wherein the medium contains lipo-derived cells of any of claims 1-7.

5 64. The method of claim 63, wherein a stem cell and a lipo-derived cell are in contact.

65. The method of any of claims 60-64, wherein a stem cell is a hemopoietic stem cell.

10 66. A method of producing animal matter comprising maintaining the composition of any of claims 18-26 under conditions sufficient for the cells within the composition to expand and differentiate to form the matter.

67. The method of claim 66, wherein the matter comprises a tissue type selected from the group of tissues consisting of adipose, cartilage, heart, dermal connective tissue, blood tissue, muscle, kidney, bone, pleural, and splanchnic tissues,
15 and combinations thereof.

68. The method of claim 66 or 67, wherein the matter comprises more than one tissue type.

69. The method of any of claims 66-68, wherein the matter comprises at least a portion of an animal organ.

20 70. The method of claim 66-68, wherein the matter comprises at least a portion of an animal limb.

71. The method of any of claims 66-70, wherein the composition is maintained *in vitro*.

25 72. The method of any of claims 66-70, wherein the composition is introduced into an animal and maintained *in vivo*.

73. An implant comprising the cell of any of claims 1-7.

74. An implant comprising the population of any of 8-13.

75. An implant comprising the lipo-derived lattice of any of claims 14-16.

76. An implant comprising the composition of any of claims 17-26.

30 77. A kit for isolating stem cells from adipose tissues comprising a means for isolating adipose tissue from a patient and a means for separating stem cells from the remainder of the adipose tissue.

78. The kit of claim 77, further comprising a medium for differentiating the stem cells.

5

MANDEL & ADRIANO

United States Patent Application

COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor I hereby declare that: my residence, post office address and citizenship are as stated below next to my name; that

I verily believe I am the original, first and sole inventor (if only one name is listed below) or a joint inventor (if plural inventors are named below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: ADIPOSE-DERIVED STEM CELLS AND LATTICES

The specification of which

- a. ☒ is attached hereto
b. ☐ was filed on as application serial no. and was amended on (if applicable) (in the case of a PCT-filed application) described and claimed in international no. filed and as amended on (if any), which I have reviewed and for which I solicit a United States patent.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with Title 37, Code of Federal Regulations, § 1.56 (attached hereto).

I hereby claim foreign priority benefits under Title 35, United States Code, § 119/365 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on the basis of which priority is claimed:

- a. ☐ no such applications have been filed.
b. ☒ such applications have been filed as follows:

FOREIGN APPLICATION(S), IF ANY, CLAIMING PRIORITY UNDER 35 USC § 119			
COUNTRY	APPLICATION NUMBER	DATE OF FILING (day, month, year)	DATE OF ISSUE (day, month, year)
PCT	PCT/US00/06232	10 March 2000	14 September 2000
ALL FOREIGN APPLICATION(S), IF ANY, FILED BEFORE THE PRIORITY APPLICATION(S)			
COUNTRY	APPLICATION NUMBER	DATE OF FILING (day, month, year)	DATE OF ISSUE (day, month, year)

I hereby claim the benefit under Title 35, United States Code, § 120/365 of any United States and PCT international application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. APPLICATION NUMBER	DATE OF FILING (day, month, year)	STATUS (patented, pending, abandoned)

I hereby claim the benefit under Title 35, United States Code § 119(e) of any United States provisional application(s) listed below:

U.S. PROVISIONAL APPLICATION NUMBER	DATE OF FILING (Day, Month, Year)
60/123,711	10 March 1999
60/162,462	29 October 1999

I hereby appoint the attorneys and agents associated with Customer No. 26,941, respectively and individually, as my attorneys and agents, with full power of substitution and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith.

I hereby authorize them to act and rely on instructions from and communicate directly with the person/assignee/attorney/firm/ organization who/which first sends/sent this case to them and by whom/which I hereby declare that I have consented after full disclosure to be represented unless/until I instruct Mandel & Adriano to the contrary.

Please direct all correspondence in this case to Mandel & Adriano at the address indicated below:

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35 No. Arroyo Parkway, Suite 60
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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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Signature of Inventor 204:			Date:	

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Date:

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Signature of Inventor 203:

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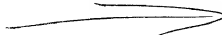
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Signature of Inventor 207: <i>Zhu Min</i>				Date: 09/06/2001

§ 1.56 Duty to disclose information material to patentability.

(a) A patent by its very nature is affected with a public interest. The public interest is best served, and the most effective patent examination occurs when, at the time an application is being examined, the Office is aware of and evaluates the teachings of all information material to patentability. Each individual associated with the filing and prosecution of a patent application has a duty of candor and good faith in dealing with the Office, which includes a duty to disclose to the Office all information known to that individual to be material to patentability as defined in this section. The duty to disclose information exists with respect to each pending claim until the claim is canceled or withdrawn from consideration, or the application becomes abandoned. Information material to the patentability of a claim that is canceled or withdrawn from consideration need not be submitted if the information is not material to the patentability of a claim remaining under consideration in the application. There is no duty to submit information which is not material to the patentability of any existing claim. The duty to disclose all information known to be material to patentability is deemed to be satisfied if all information known to be material to patentability of any claim issued in a patent was cited by the Office or submitted to the Office in the manner prescribed by §§ 1.97(b)-(d) and 1.98. However, no patent will be granted on an application in connection with which fraud on the Office was practiced or attempted or the duty of disclosure was violated through bad faith or intentional misconduct. The Office encourages applicants to carefully examine:

- (1) prior art cited in search reports of a foreign patent office in a counterpart application, and
- (2) the closest information over which individuals associated with the filing or prosecution of a patent application believe any pending claim patentably defines, to make sure that any material information contained therein is disclosed to the Office.

(b) Under this section, information is material to patentability when it is not cumulative to information already of record or being made of record in the application, and

- (1) It establishes, by itself or in combination with other information, a prima facie case of unpatentability of a claim; or
- (2) It refutes, or is inconsistent with, a position the applicant takes in:
 - (i) Opposing an argument of unpatentability relied on by the Office, or
 - (ii) Asserting an argument of patentability.

A prima facie case of unpatentability is established when the information compels a conclusion that a claim is unpatentable under the preponderance of evidence, burden-of-proof standard, giving each term in the claim its broadest reasonable construction consistent with the specification, and before any consideration is given to evidence which may be submitted in an attempt to establish a contrary conclusion of patentability.

(c) Individuals associated with the filing or prosecution of a patent application within the meaning of this section are:

(1) Each inventor named in the application;

(2) Each attorney or agent who prepares or prosecutes the application; and

(3) Every other person who is substantively involved in the preparation or prosecution of the application and who

associated with the inventor, with the assignee or with anyone to whom there is an obligation to assign the application.

(d) Individuals other than the attorney, agent or inventor may comply with this section by disclosing information to the attorney, agent, or inventor.

